

B14

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
16 January 2003 (16.01.2003)

PCT

(10) International Publication Number
WO 03/004664 A2

(51) International Patent Classification⁷: **C12P**

(21) International Application Number: PCT/EP02/06564

(22) International Filing Date: 14 June 2002 (14.06.2002)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
101 32 946.6 6 July 2001 (06.07.2001) DE
60/303,790 10 July 2001 (10.07.2001) US

CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZM, ZW.

(84) Designated States (*regional*): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

(71) Applicant (*for all designated States except US*): DE-GUSSA AG [DE/DE]; Bennigsenplatz 1, 40474 Düsseldorf (DE).

Declaration under Rule 4.17:
— of inventorship (Rule 4.17(iv)) *for US only*

(72) Inventor; and

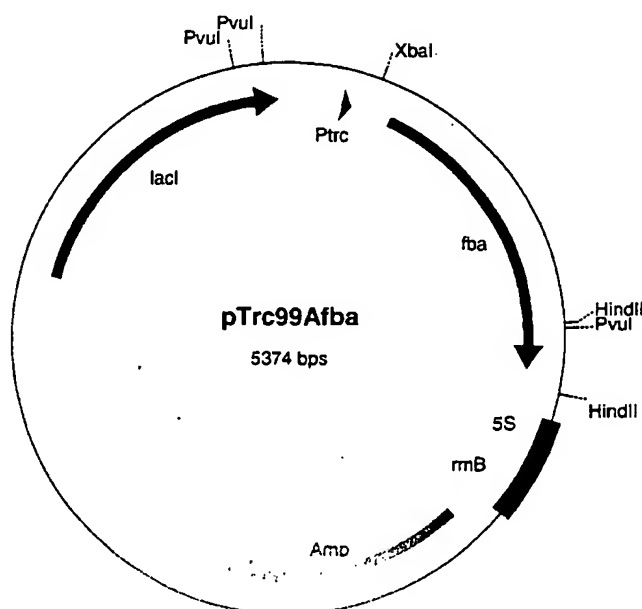
(75) Inventor/Applicant (*for US only*): RIEPING, Mechthild [DE/DE]; Mönkebergstrasse 1, 33619 Bielefeld (DE).

Published:
— without international search report and to be republished upon receipt of that report

(81) Designated States (*national*): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU,

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: PROCESS FOR THE PREPARATION OF L-AMINO ACIDS USING STRAINS OF THE ENTEROBACTERIACEAE FAMILY



(57) Abstract: The invention relates to a process for the preparation of L-amino acids, in particular L-threonine.

BEST AVAILABLE COPY



WO 03/004664 A2

**Process for the Preparation of L-Amino Acids using Strains
of the Enterobacteriaceae Family**

Field of the Invention

This invention relates to a process for the preparation of
5 L-amino acids, in particular L-threonine, using strains of
the Enterobacteriaceae family in which at least the fba
gene is enhanced.

Prior Art

L-Amino acids, in particular L-threonine, are used in human
10 medicine and in the pharmaceuticals industry, in the
foodstuffs industry and very particularly in animal
nutrition.

It is known to prepare L-amino acids by fermentation of
strains of Enterobacteriaceae, in particular Escherichia
15 coli (E. coli) and Serratia marcescens. Because of their
great importance, work is constantly being undertaken to
improve the preparation processes. Improvements to the
process can relate to fermentation measures, such as e.g.
stirring and supply of oxygen, or the composition of the
20 nutrient media, such as e.g. the sugar concentration during
the fermentation, or the working up to the product form, by
e.g. ion exchange chromatography, or the intrinsic output
properties of the microorganism itself.

Methods of mutagenesis, selection and mutant selection are
25 used to improve the output properties of these
microorganisms. Strains which are resistant to
antimetabolites, such as e.g. the threonine analogue α -
amino- β -hydroxyvaleric acid (AHV), or are auxotrophic for
metabolites of regulatory importance and produce L-amino
30 acid, such as e.g. L-threonine, are obtained in this
manner.

Methods of the recombinant DNA technique have also been employed for some years for improving the strain of strains of the Enterobacteriaceae family which produce L-amino acids, by amplifying individual amino acid biosynthesis genes and investigating the effect on the production.

Object of the invention

The object of the invention is to provide new measures for improved fermentative preparation of L-amino acids, in particular L-threonine.

Description of the invention

The invention provides a process for the preparation of L-amino acids, in particular L-threonine, using microorganisms of the Enterobacteriaceae family which in particular already produce L-amino acids and in which the nucleotide sequence which codes for the fba gene is enhanced.

Where L-amino acids or amino acids are mentioned in the following, this means one or more amino acids, including their salts, chosen from the group consisting of L-asparagine, L-threonine, L-serine, L-glutamate, L-glycine, L-alanine, L-cysteine, L-valine, L-methionine, L-isoleucine, L-leucine, L-tyrosine, L-phenylalanine, L-histidine, L-lysine, L-tryptophan and L-arginine. L-Threonine is particularly preferred.

The term "enhancement" in this connection describes the increase in the intracellular activity of one or more enzymes or proteins in a microorganism which are coded by the corresponding DNA, for example by increasing the number of copies of the gene or genes, using a potent promoter or a gene or allele which codes for a corresponding enzyme or protein with a high activity, and optionally combining these measures.

By enhancement measures, in particular over-expression, the activity or concentration of the corresponding protein is in general increased by at least 10%, 25%, 50%, 75%, 100%, 150%, 200%, 300%, 400% or 500%, up to a maximum of 1000% or 5 2000%, based on that of the wild-type protein or the activity or concentration of the protein in the starting microorganism.

The process is characterized in that the following steps are carried out:

- 10 a) fermentation of microorganisms of the Enterobacteriaceae family in which the fba gene is enhanced,
 - b) concentration of the corresponding L-amino acid in the medium or in the cells of the microorganisms of 15 the Enterobacteriaceae family, and
 - c) isolation of the desired L-amino acid, constituents of the fermentation broth and/or the biomass in its entirety or portions (> 0 to 100 %) thereof optionally remaining in the product.
- 20 The microorganisms which the present invention provides can produce L-amino acids from glucose, sucrose, lactose, fructose, maltose, molasses, optionally starch, optionally cellulose or from glycerol and ethanol. They are representatives of the Enterobacteriaceae family chosen 25 from the genera Escherichia, Erwinia, Providencia and Serratia. The genera Escherichia and Serratia are preferred. Of the genus Escherichia the species Escherichia coli and of the genus Serratia the species Serratia marcescens are to be mentioned in particular.
- 30 Suitable strains, which produce L-threonine in particular, of the genus Escherichia, in particular of the species Escherichia coli, are, for example

- Escherichia coli TF427
Escherichia coli H4578
Escherichia coli KY10935
Escherichia coli VNIIgenetika MG442
5 Escherichia coli VNIIgenetika M1
Escherichia coli VNIIgenetika 472T23
Escherichia coli BKIIM B-3996
Escherichia coli kat 13
Escherichia coli KCCM-10132.
- 10 Suitable L-threonine-producing strains of the genus
Serratia, in particular of the species Serratia marcescens,
are, for example
- Serratia marcescens HNr21
Serratia marcescens TLr156
15 Serratia marcescens T2000.
- Strains from the Enterobacteriaceae family which produce L-
threonine preferably have, inter alia, one or more genetic
or phenotypic features chosen from the group consisting of:
resistance to α -amino- β -hydroxyvaleric acid, resistance to
20 thialysine, resistance to ethionine, resistance to α -
methylserine, resistance to diaminosuccinic acid,
resistance to α -aminobutyric acid, resistance to
borrelidin, resistance to rifampicin, resistance to valine
analogues, such as, for example, valine hydroxamate,
25 resistance to purine analogues, such as, for example, 6-
dimethylaminopurine, a need for L-methionine, optionally a
partial and compensable need for L-isoleucine, a need for
meso-diaminopimelic acid, auxotrophy in respect of
threonine-containing dipeptides, resistance to L-threonine,
30 resistance to L-homoserine, resistance to L-lysine,
resistance to L-methionine, resistance to L-glutamic acid,
resistance to L-aspartate, resistance to L-leucine,
resistance to L-phenylalanine, resistance to L-serine,
resistance to L-cysteine, resistance to L-valine,
35 sensitivity to fluoropyruvate, defective threonine

- dehydrogenase, optionally an ability for sucrose utilization, enhancement of the threonine operon, enhancement of homoserine dehydrogenase I-aspartate kinase I, preferably of the feed back resistant form, enhancement
5 of homoserine kinase, enhancement of threonine synthase, enhancement of aspartate kinase, optionally of the feed back resistant form, enhancement of aspartate semialdehyde dehydrogenase, enhancement of phosphoenol pyruvate carboxylase, optionally of the feed back resistant form,
10 enhancement of phosphoenol pyruvate synthase, enhancement of transhydrogenase, enhancement of the RhtB gene product, enhancement of the RhtC gene product, enhancement of the YfiK gene product, enhancement of a pyruvate carboxylase, and attenuation of acetic acid formation.
- 15 It has been found that microorganisms of the Enterobacteriaceae family produce L-amino acids, in particular L-threonine, in an improved manner after enhancement, in particular over-expression, of the fba gene.
- 20 The use of endogenous genes is in general preferred. "Endogenous genes" or "endogenous nucleotide sequences" are understood as meaning the genes or nucleotide sequences present in the population of a species.

The nucleotide sequences of the genes of Escherichia coli
25 belong to the prior art and can also be found in the genome sequence of Escherichia coli published by Blattner et al. (Science 277: 1453-1462 (1997)).

The following information on the fba gene is known, inter alia, from the prior art:

- 30 Description: Fructose bisphosphate aldolase (class II)
EC No.: 4.1.2.13

Reference: Alefounder et al.; Biochemical Journal
257(2): 529-34 (1989) Zgiby et al.;
European Journal of Biochemistry 267(6):
1858-1868 (2000) Baldwin et al.;
5 Biochemical Journal 169(3): 633-641 (1978)
Accession No.: AE000376
Alternative gene names: fda, ald

The nucleic acid sequences can be found in the databanks of
the National Center for Biotechnology Information (NCBI) of
10 the National Library of Medicine (Bethesda, MD, USA), the
nucleotide sequence databank of the European Molecular
Biologies Laboratories (EMBL, Heidelberg, Germany or
Cambridge, UK) or the DNA databank of Japan (DDBJ, Mishima,
Japan).

15 Alleles of the fba gene which result from the degeneracy of
the genetic code or due to "sense mutations" of neutral
function can furthermore be used.

To achieve an enhancement, for example, expression of the
genes or the catalytic properties of the proteins can be
20 increased. The two measures can optionally be combined.

To achieve an over-expression, the number of copies of the
corresponding genes can be increased, or the promoter and
regulation region or the ribosome binding site upstream of
the structural gene can be mutated. Expression cassettes
25 which are incorporated upstream of the structural gene act
in the same way. By inducible promoters, it is
additionally possible to increase the expression in the
course of fermentative L-threonine production. The
expression is likewise improved by measures to prolong the
30 life of the m-RNA. Furthermore, the enzyme activity is
also enhanced by preventing the degradation of the enzyme
protein. The genes or gene constructs can either be
present in plasmids with a varying number of copies, or can
be integrated and amplified in the chromosome.

Alternatively, an over-expression of the genes in question can furthermore be achieved by changing the composition of the media and the culture procedure.

Instructions in this context can be found by the expert,
5 inter alia, in Chang and Cohen (Journal of Bacteriology 134: 1141-1156 (1978)), in Hartley and Gregori (Gene 13: 347-353 (1981)), in Amann and Brosius (Gene 40: 183-190 (1985)), in de Broer et al. (Proceedings of the National Academy of Sciences of the United States of America 80:
10 21-25 (1983)), in LaVallie et al. (BIO/TECHNOLOGY 11: 187-193 (1993)), in PCT/US97/13359, in Llosa et al. (Plasmid 26: 222-224 (1991)), in Quandt and Klipp (Gene 80: 161-169 (1989)), in Hamilton (Journal of Bacteriology 171: 4617-4622 (1989)), in Jensen and Hammer (Biotechnology and
15 Bioengineering 58: 191-195 (1998)) and in known textbooks of genetics and molecular biology.

Plasmid vectors which are capable of replication in Enterobacteriaceae, such as e.g. cloning vectors derived from pACYC184 (Bartolomé et al.; Gene 102: 75-78 (1991)),
20 pTrc99A (Amann et al.; Gene 69: 301-315 (1988)) or pSC101 derivatives (Vocke and Bastia, Proceedings of the National Academy of Sciences USA 80(21): 6557-6561 (1983)) can be used. A strain transformed with a plasmid vector, wherein the plasmid vector carries at least one nucleotide sequence
25 which codes for the fba gene, can be employed in a process according to the invention.

It is also possible to transfer mutations which affect the expression of the particular gene into various strains by sequence exchange (Hamilton et al.; Journal of Bacteriology
30 171: 4617 - 4622 (1989)), conjugation or transduction.

It may furthermore be advantageous for the production of L-amino acids, in particular L-threonine, with strains of the Enterobacteriaceae family to enhance one or more enzymes of the known threonine biosynthesis pathway or enzymes of

anaplerotic metabolism or enzymes for the production of reduced nicotinamide adenine dinucleotide phosphate, in addition to the enhancement of the fba gene.

Thus, for example, one or more of the genes chosen from the
5 group consisting of

- the thrABC operon which codes for aspartate kinase, homoserine dehydrogenase, homoserine kinase and threonine synthase (US-A-4,278,765),
- the pyc gene which codes for pyruvate carboxylase (DE-A-
10 19 831 609),
- the pps gene which codes for phosphoenol pyruvate synthase (Molecular and General Genetics 231(2): 332-336 (1992)),
- the ppc gene which codes for phosphoenol pyruvate
15 carboxylase (Gene 31: 279-283 (1984)),
- the pntA and pntB genes which code for transhydrogenase (European Journal of Biochemistry 158: 647-653 (1986)),
- the rhtB gene which imparts homoserine resistance (EP-A-0 994 190),
- the mgo gene which codes for malate:quinone
20 oxidoreductase (WO 02/06459),
- the rhtC gene which imparts threonine resistance (EP-A-1 013 765),
- the thrE gene of Corynebacterium glutamicum which codes
25 for the threonine export protein (WO 01/92545),
- the gdhA gene which codes for glutamate dehydrogenase (Nucleic Acids Research 11: 5257-5266 (1983); Gene 23: 199-209 (1983)),

- the dps gene which codes for the global regulator Dps (Genes & Development 6(12B): 2646-2654 (1992), Accession No. AE000183),
- 5 • the lrp gene which codes for the regulator of the leucine Lrp regulon and high-affinity transport systems of branched-chain amino acids (Journal of Biological Chemistry 266(17): 10768-10774 (1991), Accession No. AE000191),
- 10 • the hns gene which codes for the DNA-binding protein HLP-II (Molecular and General Genetics 212(2): 199-202 (1988), Accession No. AE000222),
- the pgm gene which codes for phosphoglucomutase (Journal of Bacteriology 176: 5847-5851 (1994), Accession No. AE000172),
- 15 • the ptsG gene which codes for the glucose-specific IIBC component of the phosphotransferase system PTS (Journal of Biological Chemistry 261(35): 16398-16403 (1986), Accession No. AE000210),
- 20 • the ptsH gene of the ptsHIcrr operon which codes for the phosphohistidine protein hexose phosphotransferase of the phosphotransferase system PTS (Journal of Biological Chemistry 262(33): 16241-16253 (1987), Accession No. AE000329),
- 25 • the ptsI gene of the ptsHIcrr operon which codes for enzyme I of the phosphotransferase system PTS (Journal of Biological Chemistry 262(33): 16241-16253 (1987), Accession No. AE000329),
- 30 • the crr gene of the ptsHIcrr operon which codes for the glucose-specific IIA component of the phosphotransferase system PTS (Journal of Biological Chemistry 262(33): 16241-16253 (1987), Accession No. AE000329),

- the mopB gene which codes for chaperone GroES (Journal of Biological Chemistry 261(26): 12414-12419 (1986), Accession No. AE000487),
- 5 • the ahpC gene of the ahpCF operon which codes for the small subunit of alkyl hydroperoxide reductase (Proceedings of the National Academy of Sciences USA 92(17): 7617-7621 (1995), Accession No. AE000166),
- 10 • the ahpF gene of the ahpCF operon which codes for the large subunit of alkyl hydroperoxide reductase (Proceedings of the National Academy of Sciences USA 92(17): 7617-7621 (1995), Accession No. AE000166),

can be enhanced, in particular over-expressed.

The use of endogenous genes is in general preferred.

15 It may furthermore be advantageous for the production of L-amino acids, in particular L-threonine, in addition to the enhancement of the fba gene, for one or more of the genes chosen from the group consisting of

- the tdh gene which codes for threonine dehydrogenase (Journal of Bacteriology 169: 4716-4721 (1987)),
- 20 • the mdh gene which codes for malate dehydrogenase (E.C. 1.1.1.37) (Archives in Microbiology 149: 36-42 (1987)),
- the gene product of the open reading frame (orf) yjfa (Accession Number AAC77180 of the National Center for Biotechnology Information (NCBI, Bethesda, MD, USA)),
- 25 • the gene product of the open reading frame (orf) ytfP (Accession Number AAC77179 of the National Center for Biotechnology Information (NCBI, Bethesda, MD, USA)),
- the pckA gene which codes for the enzyme phosphoenolpyruvate carboxykinase (Journal of Bacteriology 172: 7151-7156 (1990)),
- 30

- the poxB gene which codes for pyruvate oxidase (Nucleic Acids Research 14(13): 5449-5460 (1986)),
 - the aceA gene which codes for the enzyme isocitrate lyase (Journal of Bacteriology 170: 4528-4536 (1988)),
- 5 • the dgsA gene which codes for the DgsA regulator of the phosphotransferase system (Bioscience, Biotechnology and Biochemistry 59: 256-251 (1995)) and is also known under the name of the mlc gene,
- 10 • the fruR gene which codes for the fructose repressor (Molecular and General Genetics 226: 332-336 (1991)) and is also known under the name of the cra gene, and
- the rpoS gene which codes for the sigma³⁸ factor (WO 01/05939) and is also known under the name of the katF gene,
- 15 to be attenuated, in particular eliminated or for the expression thereof to be reduced.

The term "attenuation" in this connection describes the reduction or elimination of the intracellular activity of one or more enzymes (proteins) in a microorganism which are

20 coded by the corresponding DNA, for example by using a weak promoter or a gene or allele which codes for a corresponding enzyme with a low activity or inactivates the corresponding enzyme (protein) or gene, and optionally combining these measures.

25 By attenuation measures, the activity or concentration of the corresponding protein is in general reduced to 0 to 75%, 0 to 50%, 0 to 25%, 0 to 10% or 0 to 5% of the activity or concentration of the wild-type protein or of the activity or concentration of the protein in the

30 starting microorganism.

It may furthermore be advantageous for the production of L-amino acids, in particular L-threonine, in addition to the enhancement of the fba gene, to eliminate undesirable side reactions (Nakayama: "Breeding of Amino Acid Producing
5 Microorganisms", in: Overproduction of Microbial Products, Krumphanzl, Sikyta, Vanek (eds.), Academic Press, London, UK, 1982).

The microorganisms produced according to the invention can be cultured in the batch process (batch culture), the fed
10 batch (feed process) or the repeated fed batch process (repetitive feed process). A summary of known culture methods is described in the textbook by Chmiel (Bioprozesstechnik 1. Einführung in die Bioverfahrenstechnik [Bioprocess Technology 1. Introduction
15 to Bioprocess Technology (Gustav Fischer Verlag, Stuttgart, 1991)) or in the textbook by Storhas (Bioreaktoren und periphere Einrichtungen [Bioreactors and Peripheral Equipment] (Vieweg Verlag, Braunschweig/Wiesbaden, 1994)).

The culture medium to be used must meet the requirements of
20 the particular strains in a suitable manner. Descriptions of culture media for various microorganisms are contained in the handbook "Manual of Methods for General Bacteriology" of the American Society for Bacteriology (Washington D.C., USA, 1981).

25 Sugars and carbohydrates, such as e.g. glucose, sucrose, lactose, fructose, maltose, molasses, starch and optionally cellulose, oils and fats, such as e.g. soya oil, sunflower oil, groundnut oil and coconut fat, fatty acids, such as e.g. palmitic acid, stearic acid and linoleic acid,
30 alcohols, such as e.g. glycerol and ethanol, and organic acids, such as e.g. acetic acid, can be used as the source of carbon. These substances can be used individually or as a mixture.

Organic nitrogen-containing compounds, such as peptones, yeast extract, meat extract, malt extract, corn steep liquor, soya bean flour and urea, or inorganic compounds, such as ammonium sulfate, ammonium chloride, ammonium phosphate, ammonium carbonate and ammonium nitrate, can be used as the source of nitrogen. The sources of nitrogen can be used individually or as a mixture.

Phosphoric acid, potassium dihydrogen phosphate or dipotassium hydrogen phosphate or the corresponding sodium-containing salts can be used as the source of phosphorus. The culture medium must furthermore comprise salts of metals, such as e.g. magnesium sulfate or iron sulfate, which are necessary for growth. Finally, essential growth substances, such as amino acids and vitamins, can be employed in addition to the abovementioned substances. Suitable precursors can moreover be added to the culture medium. The starting substances mentioned can be added to the culture in the form of a single batch, or can be fed in during the culture in a suitable manner.

Basic compounds, such as sodium hydroxide, potassium hydroxide, ammonia or aqueous ammonia, or acid compounds, such as phosphoric acid or sulfuric acid, can be employed in a suitable manner to control the pH of the culture. Antifoams, such as e.g. fatty acid polyglycol esters, can be employed to control the development of foam. Suitable substances having a selective action, e.g. antibiotics, can be added to the medium to maintain the stability of plasmids. To maintain aerobic conditions, oxygen or oxygen-containing gas mixtures, such as e.g. air, are introduced into the culture. The temperature of the culture is usually 25°C to 45°C, and preferably 30°C to 40°C. Culturing is continued until a maximum of L-amino acids or L-threonine has formed. This target is usually reached within 10 hours to 160 hours.

The analysis of L-amino acids can be carried out by anion exchange chromatography with subsequent ninhydrin derivatization, as described by Spackman et al. (Analytical Chemistry 30: 1190-1206 (1958)), or it can take place by
5 reversed phase HPLC as described by Lindroth et al. (Analytical Chemistry 51: 1167-1174 (1979)).

The process according to the invention is used for the fermentative preparation of L-amino acids, such as, for example, L-threonine, L-isoleucine, L-valine, L-methionine,
10 L-homoserine and L-lysine, in particular L-threonine.

The present invention is explained in more detail in the following with the aid of embodiment examples.

The minimal (M9) and complete media (LB) for Escherichia coli used are described by J.H. Miller (A Short Course in
15 Bacterial Genetics (1992), Cold Spring Harbor Laboratory Press). The isolation of plasmid DNA from Escherichia coli and all techniques of restriction, ligation, Klenow and alkaline phosphatase treatment are carried out by the method of Sambrook et al. (Molecular Cloning - A Laboratory
20 Manual (1989) Cold Spring Harbor Laboratory Press). Unless described otherwise, the transformation of Escherichia coli is carried out by the method of Chung et al. (Proceedings of the National Academy of Sciences of the United States of America 86: 2172-2175 (1989)).

25 The incubation temperature for the preparation of strains and transformants is 37°C.

Example 1

Construction of the expression plasmid pTrc99Afba

The fba gene from E. coli K12 is amplified using the
30 polymerase chain reaction (PCR) and synthetic oligonucleotides. Starting from the nucleotide sequence of the fba gene in E. coli K12 MG1655 (Genbank accession

AE000376, Blattner et al. (Science 277, 1453 - 1462 (1997)), PCR primers are synthesized (MWG Biotech, Ebersberg, Germany):

fba1: 5' - CGACGATACAGGACAAGAGAC - 3' (SEQ ID No. 1)

5 fba2: 5' - GCAGAAAGGAATATCTTACAG - 3' (SEQ ID No. 2)

The chromosomal E. coli K12 MG1655 DNA employed for the PCR is isolated according to the manufacturer's instructions with "Qiagen Genomic-tips 100/G" (QIAGEN, Hilden, Germany). A DNA fragment approx. 1100 bp in size can be amplified
10 with the specific primers under standard PCR conditions (Innis et al. (1990) PCR Protocols. A Guide to Methods and Applications, Academic Press) with Pfu-DNA polymerase (Promega Corporation, Madison, USA). The PCR product is
15 PCR Cloning Kit, Invitrogen, Groningen, The Netherlands) in accordance with the manufacturer's instructions and transformed in the E. coli strain TOP10. Selection for plasmid-carrying cells is carried out on LB agar, to which 50 µg/ml kanamycin are added. After isolation of the
20 plasmid DNA, the vector pCR-Blunt II-TOPO-fba is cleaved with the restriction enzymes SpeI and XbaI and, after separation in 0.8% agarose gel with the aid of the QIAquick Gel Extraction Kit (QIAGEN, Hilden, Germany), the fba fragment is isolated. The vector pTrc99A (Pharmacia
25 Biotech, Uppsala, Sweden) is cleaved with the enzyme XbaI and ligated with the isolated fba fragment. The E. coli strain XL1-Blue MRF' (Stratagene, La Jolla, USA) is transformed with the ligation batch and plasmid-carrying cells are selected on LB agar, to which 50 µg/ml ampicillin
30 are added. Successful cloning can be demonstrated after plasmid DNA isolation by control cleavage with the enzymes HindIII and PvuII. The plasmid is called pTrc99Afba (figure 1).

Example 2

Preparation of L-threonine with the strain MG442/pTrc99Afba

The L-threonine-producing E. coli strain MG442 is described in the patent specification US-A- 4,278,765 and deposited
5 as CMIM B-1628 at the Russian National Collection for Industrial Microorganisms (VKPM, Moscow, Russia).

The strain MG442 is transformed with the expression plasmid pTrc99Afba described in example 1 and with the vector pTrc99A and plasmid-carrying cells are selected on LB agar
10 with 50 µg/ml ampicillin. The strains MG442/pTrc99Afba and MG442/pTrc99A are formed in this manner. Selected individual colonies are then multiplied further on minimal medium with the following composition: 3.5 g/l $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, 1.5 g/l KH_2PO_4 , 1 g/l NH_4Cl , 0.1 g/l $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 2 g/l
15 glucose, 20 g/l agar, 50 mg/l ampicillin. The formation of L-threonine is checked in batch cultures of 10 ml contained in 100 ml conical flasks. For this, 10 ml of preculture medium of the following composition: 2 g/l yeast extract, 10 g/l $(\text{NH}_4)_2\text{SO}_4$, 1 g/l KH_2PO_4 , 0.5 g/l $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 15 g/l
20 CaCO_3 , 20 g/l glucose, 50 mg/l ampicillin are inoculated and the batch is incubated for 16 hours at 37°C and 180 rpm on an ESR incubator from Kühner AG (Birsfelden, Switzerland). 250 µl portions of this preculture are transinoculated into 10 ml of production medium (25 g/l
25 $(\text{NH}_4)_2\text{SO}_4$, 2 g/l KH_2PO_4 , 1 g/l $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.03 g/l $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.018 g/l $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 30 g/l CaCO_3 , 20 g/l glucose, 50 mg/l ampicillin) and the batch is incubated for 48 hours at 37°C. The formation of L-threonine by the starting strain MG442 is investigated in the same manner,
30 but no addition of ampicillin to the medium takes place. After the incubation the optical density (OD) of the culture suspension is determined with an LP2W photometer from Dr. Lange (Düsseldorf, Germany) at a measurement wavelength of 660 nm.

The concentration of L-threonine formed is then determined in the sterile-filtered culture supernatant with an amino acid analyzer from Eppendorf-BioTronik (Hamburg, Germany) by ion exchange chromatography and post-column reaction
5 with ninhydrin detection.

The result of the experiment is shown in Table 1.

Table 1

Strain	OD (660 nm)	L-Threonine g/l
MG442	5.6	1.4
MG442/pTrc99A	3.8	1.3
MG442/pTrc99Afba	5.9	2.6

Brief Description of the Figure:

- 10 • Figure 1: Map of the plasmid pTrc99Afba containing the fba gene.

The length data are to be understood as approx. data. The abbreviations and designations used have the following meaning:

- 15 • Amp: Ampicillin resistance gene
- lacI: Gene for the repressor protein of the trc promoter
- Ptrc: trc promoter region, IPTG-inducible
- fba: Coding region of the fba gene
- 20 • 5S: 5S rRNA region
- 3'UTR: 3'UTR region

The abbreviations for the restriction enzymes have the following meaning

- HindIII: Restriction endonuclease from *Haemophilus influenzae* Rd
- 5 • PvuII: Restriction endonuclease from *Proteus vulgaris*
- SpeI: Restriction endonuclease from *Sphaerotilus* species ATCC 13923
- XbaI: Restriction endonuclease from *Xanthomonas campestris*

What is claimed is:

1. Process for the preparation of L-amino acids, in particular L-threonine, wherein the following steps are carried out:
 - 5 a) fermentation of microorganisms of the Enterobacteriaceae family which produce the desired L-amino acid and in which the fba gene or the nucleotide sequence which codes for this is enhanced, in particular over-expressed,
 - 10 b) concentration of the desired L-amino acid in the medium or in the cells of the microorganisms, and
 - c) isolation of the desired L-amino acid, constituents of the fermentation broth and/or the biomass in its entirety or portions (> 0 to 100%) thereof
 - 15 optionally remaining in the product.
2. Process according to claim 1, wherein microorganisms in which further genes of the biosynthesis pathway of the desired L-amino acid are additionally enhanced are employed.
- 20 3. Process according to claim 1, wherein microorganisms in which the metabolic pathways which reduce the formation of the desired L-amino acid are at least partly eliminated are employed.
4. Process according to claim 1, wherein the expression of
- 25 the polynucleotide which codes for the fba gene is increased.
5. Process according to claim 1, wherein the regulatory and/or catalytic properties of the polypeptide (protein) for which the polynucleotide fba codes are
- 30 improved or increased.

6. Process according to claim 1, wherein, for the preparation of L-amino acids, microorganisms of the Enterobacteriaceae family in which in addition at the same time one or more of the genes chosen from the group consisting of:
- 5
- 6.1 the thrABC operon which codes for aspartate kinase, homoserine dehydrogenase, homoserine kinase and threonine synthase,
- 6.2 the pyc gene which codes for pyruvate carboxylase,
- 10
- 6.3 the pps gene which codes for phosphoenolpyruvate synthase,
- 6.4 the ppc gene which codes for phosphoenolpyruvate carboxylase,
- 15
- 6.5 the pntA and pntB genes which code for transhydrogenase,
- 6.6 the rhtB gene which imparts homoserine resistance,
- 6.7 the mqo gene which codes for malate:quinone oxidoreductase,
- 20
- 6.8 the rhtC gene which imparts threonine resistance,
- 6.9 the thrE gene which codes for the threonine export protein,
- 25
- 6.10 the gdhA gene which codes for glutamate dehydrogenase,
- 6.11 the dps gene which codes for the global regulator Dps,

- 6.12 the lrp gene which codes for the regulator of the leucine Lrp regulon,
- 6.13 the hns gene which codes for the DNA-binding protein HLP-II
- 5 6.14 the pgm gene which codes for phosphoglucomutase,
- 6.15 the ptsG gene which codes for the glucose-specific IIBC component,
- 10 6.16 the ptsH gene which codes for the phosphohistidine protein hexose phosphotransferase,
- 6.17 the ptsI gene which codes for enzyme I of the phosphotransferase system,
- 15 6.18 the crr gene which codes for the glucose-specific IIA component,
- 6.19 the mopB gene which codes for chaperone GroES,
- 6.20 the ahpC gene which codes for the small sub-unit of alkyl hydroperoxide reductase,
- 20 6.21 the ahpF gene which codes for the large sub-unit of alkyl hydroperoxide reductase,

is or are enhanced, in particular over-expressed, are fermented.

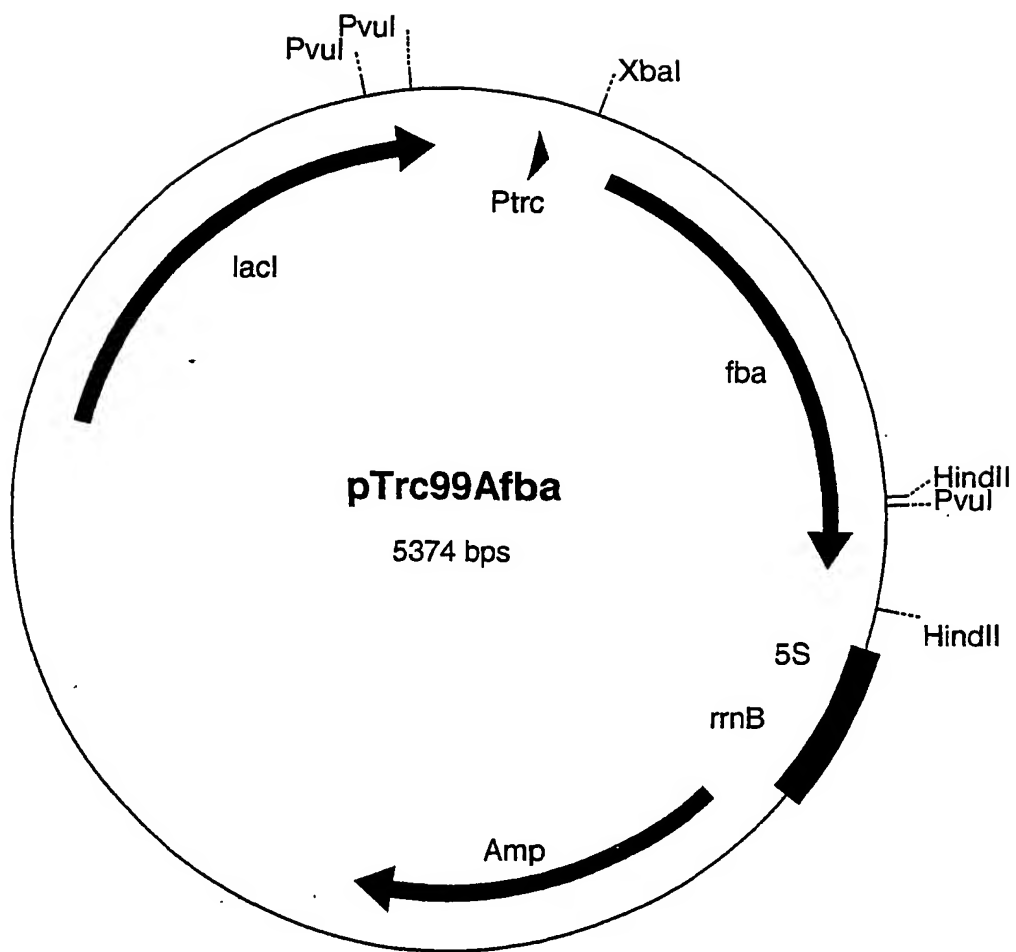
7. Process according to claim 1, wherein, for the preparation of L-amino acids, microorganisms of the Enterobacteriaceae family in which in addition at the same time one or more of the genes chosen from the group consisting of:
- 25

- 7.1 the tdh gene which codes for threonine dehydrogenase,

- 7.2 the mdh gene which codes for malate dehydrogenase,
- 7.3 the gene product of the open reading frame (orf) yjfa,
- 5 7.4 the gene product of the open reading frame (orf) ytfp,
- 7.5 the pckA gene which codes for phosphoenolpyruvate carboxykinase,
- 7.6 the poxB gene which codes for pyruvate oxidase,
- 10 7.7 the aceA gene which codes for isocitrate lyase,
- 7.8 the dgsA gene which codes for the DgsA regulator of the phosphotransferase system,
- 7.9 the fruR gene which codes for the fructose repressor,
- 15 7.10 the rpoS gene which codes for the sigma³⁸ factor

is or are attenuated, in particular eliminated or reduced in expression, are fermented.

Figure 1:



SEQUENCE PROTOCOL

5 <110> Degussa AG
<120> Process for the preparation of L-amino acids using
strains of the Enterobacteriaceae family
10 <130> 020097 BT
<160> 2
<170> PatentIn version 3.1
15 <210> 1
<211> 21
<212> DNA
<213> artificial sequence
20 <220>
<221> Primer
<222> (1)..(21)
<223> fba1
25 <400> 1
cgacgatata ggacaagaga c 21
<210> 2
<211> 21
30 <212> DNA
<213> artificial sequence
<220>
<221> Primer
35 <222> (1)..(21)
<223> fba2
<400> 2
gcagaaagga atatcttaca g 21
40

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
16 January 2003 (16.01.2003)

PCT

(10) International Publication Number
WO 03/004664 A3

(51) International Patent Classification⁷: C12N 15/31,
C12P 13/08

(21) International Application Number: PCT/EP02/06564

(22) International Filing Date: 14 June 2002 (14.06.2002)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
101 32 946.6 6 July 2001 (06.07.2001) DE
60/303,790 10 July 2001 (10.07.2001) US

(71) Applicant (for all designated States except US): DE-
GUSSA AG [DE/DE]; Bennigsenplatz 1, 40474 Düssel-
dorf (DE).

(72) Inventor; and

(75) Inventor/Applicant (for US only): RIEPING, Mechthild
[DE/DE]; Mönkebergstrasse 1, 33619 Bielefeld (DE).

(81) Designated States (national): AE, AG, AL, AM, AT, AU,
AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU,
CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH,

GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC,
LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW,
MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG,
SI, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ,
VN, YU, ZA, ZM, ZW.

(84) Designated States (regional): ARIPO patent (GH, GM,
KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW),
Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM),
European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR,
GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent
(BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR,
NE, SN, TD, TG).

Declaration under Rule 4.17:

— of inventorship (Rule 4.17(iv)) for US only

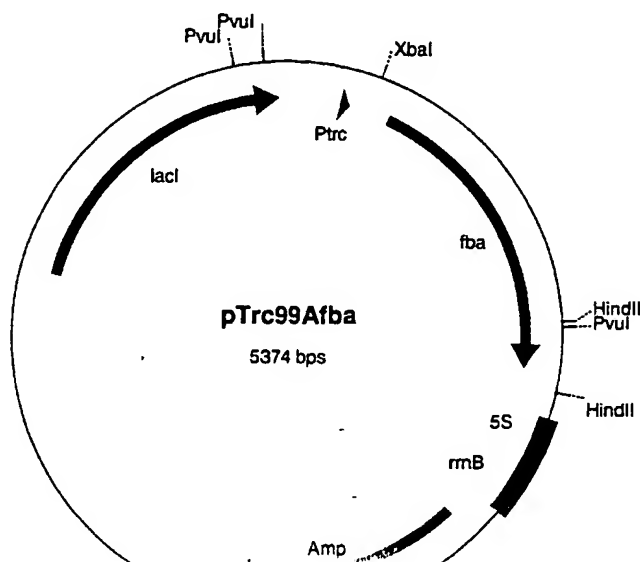
Published:

— with international search report

(88) Date of publication of the international search report:
31 July 2003

For two-letter codes and other abbreviations, refer to the "Guid-
ance Notes on Codes and Abbreviations" appearing at the begin-
ning of each regular issue of the PCT Gazette.

(54) Title: PROCESS FOR THE PREPARATION OF L-AMINO ACIDS USING STRAINS OF THE ENTEROBACTERIACEAE
FAMILY WITH ENHANCED FBA EXPRESSION



03/004664 A3

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 02/06564

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/31 C12P13/08

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N C12P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the International search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 4 278 765 A (DEBBOV VLADIMIR G ET AL) 14 July 1981 (1981-07-14) cited in the application the whole document	1-7
A	--- BABUL JORGE ET AL: "Glucose metabolism in Escherichia coli and the effect of increased amount of aldolase." BIOCHEMISTRY, vol. 32, no. 17, 1993, pages 4685-4692, XP002238441 ISSN: 0006-2960 the whole document	1-7
A	--- EP 0 994 190 A (AJINOMOTO KK) 19 April 2000 (2000-04-19) the whole document	1-7

	-/-	

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents:

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the international filing date
- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

- *T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- *X* document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- *Y* document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- *G* document member of the same patent family

Date of the actual completion of the international search

16 April 2003

Date of mailing of the international search report

06/05/2003

Fax: (+31-70) 340-3016

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 02/06564

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	SAWERS G: "THE ANAEROBIC DEGRADATION OF L-SERINE AND L-THREONINE IN ENTEROBACTERIA: NETWORKS AND PATHWAYS AND REGULATORY SIGNALS" ARCHIVES OF MICROBIOLOGY, BERLIN, DE, vol. 171, no. 1, 1998, pages 1-5, XP002953871 ISSN: 0302-8933 the whole document ---	1-7
E	WO 03 004598 A (RIEPING MECHTHILD ;DEGUSSA (DE)) 16 January 2003 (2003-01-16) claim 6 & WO 03 004663 A 16 January 2003 (2003-01-16) & WO 03 004665 A & WO 03 004669 A & WO 03 004670 A & WO 03 004671 A & WO 03 004674 A & WO 03 004675 A ---	6
T	WO 03 004662 A (DEGUSSA) 16 January 2003 (2003-01-16) the whole document ---	1-7
T	WO 02 074966 A (DEGUSSA) 26 September 2002 (2002-09-26) the whole document ---	
T	WO 02 101054 A (DEGUSSA) 19 December 2002 (2002-12-19) the whole document -----	

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/EP 02/06564

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
US 4278765	A	14-07-1981	SU 875663 A1 HU 190999 B	15-09-1982 28-12-1986
EP 0994190	A	19-04-2000	RU 2144564 C1 AU 4755099 A BR 9904955 A CN 1254014 A EP 0994190 A2 JP 2000116390 A KR 2000029006 A SK 140899 A3 US 6303348 B1 US 2002102670 A1 US 2002058314 A1 ZA 9906042 A	20-01-2000 20-04-2000 12-12-2000 24-05-2000 19-04-2000 25-04-2000 25-05-2000 16-05-2000 16-10-2001 01-08-2002 16-05-2002 04-04-2000
WO 03004598	A	16-01-2003	DE 10132946 A1 WO 03004663 A2 WO 03004669 A2 WO 03004674 A2 WO 03004670 A2 WO 03004664 A2 WO 03004598 A2 WO 03004665 A2 WO 03004671 A2 WO 03004675 A2	16-01-2003 16-01-2003 16-01-2003 16-01-2003 16-01-2003 16-01-2003 16-01-2003 16-01-2003 16-01-2003 16-01-2003
WO 03004662	A	16-01-2003	DE 10132945 A1 WO 03004662 A2 US 2003017556 A1	16-01-2003 16-01-2003 23-01-2003
WO 02074966	A	26-09-2002	DE 10113011 A1 WO 02074966 A2	19-09-2002 26-09-2002
WO 02101054	A	19-12-2002	DE 10128780 A1 WO 02101068 A2 WO 02101062 A2 WO 02101063 A2 WO 02101064 A2 WO 02101054 A2 WO 02101065 A2 WO 02101066 A2 WO 02101055 A2 WO 02101050 A2 WO 02101067 A2 US 2003064485 A1	19-12-2002 19-12-2002 19-12-2002 19-12-2002 19-12-2002 19-12-2002 19-12-2002 19-12-2002 19-12-2002 19-12-2002 19-12-2002 03-04-2003

**This Page is Inserted by IFW Indexing and Scanning
Operations and is not part of the Official Record**

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

☐ **BLACK BORDERS**

☐ **IMAGE CUT OFF AT TOP, BOTTOM OR SIDES**

☒ **FADED TEXT OR DRAWING**

☐ **BLURRED OR ILLEGIBLE TEXT OR DRAWING**

☐ **SKEWED/SLANTED IMAGES**

☐ **COLOR OR BLACK AND WHITE PHOTOGRAPHS**

☐ **GRAY SCALE DOCUMENTS**

☐ **LINES OR MARKS ON ORIGINAL DOCUMENT**

☐ **REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY**

☐ **OTHER:** _____

IMAGES ARE BEST AVAILABLE COPY.

As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.